Institut für Veterinärpathologie
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. Andreas Pospischil

A Comprehensive Test System to Identify Suitable Antibodies Against p53 for Immunohistochemical Analysis of Canine Tissues

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vorgelegt von

Stefan Keller

Tierarzt
von Karlsruhe, Deutschland

genehmigt auf Antrag von
Prof. Dr. Franco Guscetti, Referent
Prof. Dr. Hanspeter Nägeli, Korreferent

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A Comprehensive Test System to Identify Suitable Antibodies against p53 for Immunohistochemical Analysis of Canine Tissues

Summary

The tumour suppressor p53 is commonly detected in tissues of companion animals by means of antibodies raised against the human orthologue. The following three-step procedure was devised to test the suitability of such antibodies for immunohistochemistry on canine tissues. (1) Western blot and immunohistochemical analyses on bacterially expressed recombinant canine protein to assess human-to-canine cross-reactivity. (2) Immunohistochemistry of cultured, UVB-irradiated canine keratinocytes to evaluate suitability for detection of endogenous p53. (3) Immunohistochemistry on tissue arrays to further substantiate suitability of the antibodies on a panel of normal and neoplastic human and canine tissues. Five of six antibodies cross-reacted with recombinant canine p53. Three of these (PAb122, PAb240, CM-1) also immunohistochemically recognized stabilized wild type p53 in cell cultures and elicited a consistent, characteristic labelling pattern in a subset of tumours. However, two alternative batches of polyclonal antibody CM-1 failed to detect p53 in cell cultures, while showing a characteristic labelling pattern of a completely different subset of tumours and unspecific labelling of normal tissues. The test system described is well suited to the selection of antibodies for immunohistochemical p53 detection. The results emphasize the need to include appropriate controls, especially for polyclonal antibodies.

Introduction

The tumour suppressor p53 is of pivotal importance for the control of cell cycle arrest, DNA repair, and apoptosis following DNA damage (Chipuk and Green, 2006). Mutations of the p53 gene are frequent in human cancer (Vogelstein *et al*., 2000).

The mutated protein displays altered function and has a prolonged half-life, which leads to its intranuclear accumulation and renders it detectable by immunohistochemistry. This technique is widely used to screen for mutated p53 due to its simplicity, relatively high throughput and low cost as compared with mutational analysis, which is in turn more reliable and more informative (Soussi and Beroud, 2001). Numerous human studies have shown that p53 immunoreactivity is correlated with poor prognosis in several different types of tumour, while other studies have failed to confirm such correlation (Kirsch and Kastan, 1998; Peller, 1998). Analysis of the data available from the literature on human p53 indicates lack of standardization as a major source of discrepancies between different laboratories (Soussi and Beroud, 2001; Munro *et al*., 2005). Immunohistochemical assessment of the p53 status has been performed for several different types of canine tumours to determine its importance in tumorigenesis (Table 1), its possible correlation with parameters associated with biological behaviour, or its prognostic significance (Sagartz *et al*., 1996; Wolf *et al*., 1997; Ginn *et al*., 2000; Jaffe *et al*., 2000; Loukopoulos *et al*., 2003).

Most commercially available antibodies are raised against human- or mouse-specific immunogens. Use of such antibodies on canine tissues is associated with potential problems, including lack of interspecies cross-reactivity due to epitope sequence differences or insufficient specificity due to cross-reaction with unrelated antigens. A number of different antibodies have been used for the immunohistochemical detection of p53 in canine tissues (reviewed in Table 1). Most studies rely on CM-1, a polyclonal antibody raised against full-length recombinant human p53. Clone PAb240, is the second most commonly used antibody. In addition, the use of at least 11 further antibodies has been reported, mainly in single studies and with small numbers of samples. In the publications listed in Table 1, positive controls consisted mainly of human positive tissues (seven studies), of canine positive tumour tissues (three studies) or both (three studies). Paraffin wax-embedded canine cell cultures transformed with SV40 large T antigen to enforce p53 stabilization were used in four studies; in reports of 10 studies, positive controls were not mentioned. Negative controls included either omission of the primary antibody (five studies) or use of non-immune serum instead of the primary antibody (nine studies), variably combined with the use of internal tissue controls (10 studies) or of normal tissues (five studies). Reports of 11 studies made no mention of negative controls.

The purpose of the present study was to devise a system for selecting commercially available antibodies for immunohistochemical detection of canine p53.

Table 1: Antibodies against p53 and use in immunohistochemistry of canine tumours

Antibody	Immunogen/ epitope*	Species of immunogen	Homology, human-canine†	References to canine tumours
BP53-12	19-25	human	5,6/7	**Haga *et al*., 2001**
DO-1	20-25	human	4,5/6	**Johnston *et al*., 1996**; Kawaura *et al*., 2001; **Roels *et al*., 2001**
DO-7	20-25	human	4,5/6	**Albaric *et al*., 2001**
DO-12	256-270	human	14,15/15	no reference
DO-13	26-35	human	8,10/10	no reference
PAb122	370-378	human	8,9/9	**Haga *et al*., 2001**
PAb240	213-217	human	5/5	**Albaric *et al*., 2001**; **Johnston *et al*., 1996**; **Lee *et al*., 2004**; **Roels *et al*., 2001**; Teifke and Lohr, 1996; Teifke *et al*., 1998
PAb246	88-109	mouse	17,17/22	**Inoue and Wada, 2000**
PAb421	371-380	human	9,10/10	**Lee *et al*., 2004**
PAb1801	46-55	human	2,4/10	Inoue and Shiramizu, 1999; **Johnston *et al*., 1996**
CM-1	full length	human	318,341/394	**Albaric *et al*., 2001**; Gamblin *et al*., 1997; Ginn *et al*., 2000; Jaffe *et al*., 2000; Loukopoulos *et al*., 2003; McEntee and Brenneman, 1999; Murakami *et al*., 2000; Nieto *et al*., 2003; Ozaki *et al*., 2002; Pena *et al*., 2003; **Roels *et al*., 2001**; Rungsipipat *et al*., 1999; Sagartz *et al*., 1996; Schafer *et al*., 1998; Sokolowska *et al*., 2005; Sueiro *et al*., 2004; Wakui *et al*., 2001; Wolf *et al*., 1997
FL-393	full length	human	318,341/394	**Inoue and Wada, 2000**
NCD		NCD	NCD	Vitellozzi *et al*., 1998; Stoica *et al*., 2004

* Amino-acid position of human p53.

† A,B/C; A, number of identical amino acids; B, number of similar amino acids; C, total number of amino acids.

References in bold print indicate studies in which several antibodies were used.

NCD, not clearly defined.

Materials and Methods

Primary Antibodies for Immunohistochemistry

Antibodies against p53. Based on the canine p53 protein reference sequence available from Genbank (NP_001003210) and on the sequence of our own clone, we identified commercially available antibodies raised against peptides displaying the highest sequence homology possible with the canine orthologue. A further selection criterion was previous successful use according to the literature (Table 1). The following antibodies against p53 were included: two rabbit polyclonal antibodies (both CM-1; Novocastra, Newcastle upon Tyne, UK, Lot No. 300507 and 300509; Signet Laboratories, Dedham, MA, USA, Lot No. 05DC00654 and 05LC02510), five mouse monoclonal antibodies (clone DO-7, Dako Cytomation, Glostrup, Denmark; clone DO-12, Serotec, Oxford, UK; clone DO-13, Serotec; clone PAb122, Monosan, Uden, Netherlands; clone PAb240, Dako).

Antibodies against other molecules. A rabbit polyclonal antibody specific for cleaved caspase-3 (AF835, R&D Systems, Abingdon, UK) was used. This antibody is directed against an epitope adjacent to the human caspase-3 cleavage site, which is identical with the corresponding canine sequence.

Cloning of p53 and Expression in *Escherichia coli*

Cloning of the p53 open reading frame (ORF) will be described in detail elsewhere (Schade *et al*., to be published). Briefly, total RNA was isolated from Madin Darby Canine Kidney (MDCK) cells with the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Then 40 U of the Protector RNAse Inhibitor (Roche, Mannheim, Germany) were added to the extract. Complementary DNA was synthesized from 1 µg total RNA with an oligo-dT-primer and the first strand cDNA Synthesis Kit for RT-PCR (AMV) according to the instructions of the manufacturer (Roche).

The p53 ORF was amplified by the PCR with primers p53CExUp (5'-atgaattcctgcgatgcaagagccac-3') and p53CExDn (5'-taatgtcgacgaagtcagtctgagtcggg-3') and with the “Platinum Taq DNA Polymerase High Fidelity” (Invitrogen, Carlsbad, CA, USA), and cloned by sticky-end ligation into the inducible glutathione S-transferase (GST)-tagged pGEX-4T2 bacterial expression vector (Invitrogen). Vectors were transformed into DH5a competent cells (Invitrogen) and individual

clones sequenced (Microsynth AG, Balgach, Switzerland). A clone containing the correct p53 sequence was transformed into BL-21 Star *E. coli* (Invitrogen) for protein expression. Cultures of bacteria containing the GST-p53 fusion construct and the empty vector coding for GST alone were grown to an OD_{600} of 0.5 – 0.8 in LB medium (Oxoid AG, Pratteln, Switzerland) supplemented with ampicillin. Protein expression was induced by adding 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG). The bacteria were harvested after 2.5 h of further growth under the same conditions and processed either for Western blotting or for paraffin wax embedding.

Western Blots

Bacteria were pelleted by centrifugation of 1-ml cultures. The supernate was discarded and the pellets were resuspended in 200 µl of 2x sodium dodecyl sulphate (SDS) buffer containing β-mercaptoethanol 10%. After incubation for 10 min at 95°C, 10 to 20 µl of bacterial lysates were loaded on 8% SDS polyacrylamide gels and separated by gel electrophoresis (SDS-PAGE). The proteins were transferred to a nitrocellulose membrane by means of a semi-dry blotting system (Biometra, Goettingen, Germany). Non-specific antibody binding sites were blocked by incubation of the membrane for 1 h at room temperature in Tris-buffered saline containing Tween 20 0.05% (TBST) and with milk powder 4%. After washing for 1x 15 min and 2x 5 min in TBST, the membrane was incubated for 1 h at room temperature with primary antibodies DO-12, DO-13, PAb122, PAb240 and CM-1 (Novocastra, Lot No. 300507) diluted 1 in 500 in TBST containing BSA 3%. The secondary antibodies (Goat-anti-mouse-HRP [Geno Technologies, St Louis, MO, USA] diluted 1 in 10 000 and anti-rabbit-HRP [Jackson ImmunoResearch, Soham, UK] diluted 1 in 20 000) were applied for 1 h at room temperature in TBST containing BSA 3%. The signal was detected by incubating the membrane with Lumi Glo (KPL, Maryland, USA) following the instructions of the supplier.

Cell Culture and Ultraviolet B (UVB) Irradiation

Normal canine keratinocyte cultures (passage 18) have been described previously (Kolly *et al*., 2005). The cells were grown in 5% CO_2 at 37°C in Dulbeccos' Modified Eagles medium supplemented with non-essential amino acids 1%, sodium pyruvate 1%, penicillin/streptomycin 1% and fetal calf serum 15% (all cell culture reagents from Gibco BRL Life Sciences, Basel, Switzerland).

To induce apoptosis, the cells were irradiated at a dose of 100 mJ/cm^2 UVB with a DNA crosslinker (ltf Labortechnik, Wasserburg, Germany) with an emission peak at 312 nm. Four plates were irradiated at different timepoints, each with a single dose. The first time point was at 24 h after plating, when cells had reached about 50% confluence. The subsequent irradiations followed at 6-h intervals, the final dose being delivered 42 h after plating. During irradiation, the culture medium was replaced with phosphate-buffered saline (PBS); thereafter the cells were further incubated in culture medium. All cultures were harvested simultaneously 48 h after plating and were further processed together. Cells not exposed to UV irradiation but washed with PBS served as negative controls. The cells were either grown in 60 mm^2 culture dishes for paraffin wax embedding or in 24 well plates containing coverslips for Giemsa staining.

Giemsa Staining

The cells were rinsed four times with PBS, fixed with methanol for 10 min, and stained with Giemsa (1 in 10 in distilled water) for 10 min. After a brief treatment with acetic acid and isopropanol, the coverslips were mounted on glass slides (Erie Scientific Company, Portsmouth, NH, USA) with Shandon-Mount (Thermo Electron Corporation, Waltham, MA, USA).

Fluorescence-Activated Cell Sorting (FACS) Analysis

Normal keratinocytes were irradiated with a single dose of UVB (100 mJ/cm^2) 24 h after plating as described above, or left untreated (control). Twenty-four hours later the cells were rinsed twice with PBS and trypsinized. After centrifugation (2 min at 600***g***) cell pellets were resuspended in Annexin V binding buffer (10mM Hepes/NaOH, 140 mM NaCl and 2.5 mM $CaCl_2$) containing ethidium bromide (EtBr) 1 μg/ml and Annexin V FITC 5% (BD Pharmingen, Basel, Switzerland). The samples were incubated for 15 min at room temperature and subsequently analysed by flow cytometry (FACSCalibur apparatus with BD CellQuestTM Pro software (BD Pharmingen, Becton Dickinson).

Fixation and Embedding of Bacteria and Keratinocytes

Bacterial culture (100 ml) was centrifuged at 4000***g*** for 10 min at 4°C. The supernate was discarded and the pellets were resuspended in 4% neutral buffered

formaldehyde and incubated for 24 h at room temperature. Subsequently, the cultures were centrifuged at 4000***g*** for 10 min at room temperature and the pellets were resuspended in 1.5 ml of BSA 5% in PBS and transferred into Eppendorf tubes. After centrifugation at 13 000 rpm at room temperature for 10 min the pellets were embedded in paraffin wax by the Cytoblock system (Thermo Shandon, Pittsburgh, PA, USA) and a routine embedding procedure.

Keratinocytes were fixed in 4% neutral buffered formaldehyde for 1 h and then harvested with a cell scraper. After centrifugation for 5 min at 3000***g*** and two washes with PBS, the supernate was discarded and the pellets were resuspended in 1.5 ml of bovine serum albumin 5% in PBS and transferred to Eppendorf tubes. The cells were then centrifuged at 3000***g*** for 10 min and the resulting pellets were paraffin wax-embedded by the Cytoblock system (Thermo Shandon, Pittsburgh, PA, USA) as above.

Tissue Array Construction

Tissue arrays were constructed from archival samples by means of a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). They included human tumour tissues with known immunohistochemical p53 status (one positive and two negative colon carcinomas; two positive and one negative breast carcinomas) and canine normal and neoplastic tissues. The canine normal tissues consisted of all major organs, each derived from three different animals and were free of histopathological lesions. Samples included stomach, duodenum, jejunum, colon, spleen, tonsil, lymph node, thymus, liver, kidney, thyroid gland, skeletal muscle, brain, spinal cord, ovary, uterus, vagina, mammary gland, prostate, testis, heart, lung and trachea. The canine tumour tissues consisted of specimens of seminoma (five), fibrosarcoma (five), osteosarcoma (four) and mammary adenocarcinoma (four). Also included were 24 malignant lymphomas, in view of the contradictory published data on the expression of p53 by this tumour type (Gamblin *et al*., 1997; Sueiro *et al*., 2004; Sokolowska *et al*., 2005). Cylinders of 1.2 mm diameter (one for each normal organ and two to four for each of the tumour samples) were collected from representative areas of each specimen and transferred to paraffin wax recipient blocks (Micro-Cut Paraffin, Polysciences Inc., Warrington, PA, USA). All specimens had previously been fixed in 4% buffered formaldehyde and paraffin wax-embedded. Before sectioning, the blocks

were immersed in water and kept at -20 °C for 15 min. Sections (1.5 μm) were transferred to positively charged glass slides (Erie Scientific Company).

Immunohistochemistry (IHC)

Immunohistochemistry procedures were optimized for p53 antibody usage as follows. All antibodies were first applied according to the manufacturer's instructions. If the recommended procedure did not elicit the expected diffuse intranuclear signal with human and selected canine tumours and cell pellets, different antigen retrieval methods, as well as different dilutions and incubation conditions for the primary antibody, were tested. The slides were either microwaved in acidic (citrate buffer, pH 6.0, Dako Cytomation) or alkaline buffer (Tris/EDTA buffer, pH 9.0, Dako Cytomation) or treated with pronase (0.05%; Dako Cytomation). The antibody dilution was decreased to as little as 1 in 10 or until non-specific staining became dominant. Incubation with the primary antibody was carried out either overnight at 4 °C or for 1 h at room temperature. The chromogen 3-amino-9-ethyl-carbazole (AEC) was applied for a variable period up to a maximum of 30 min. If an antibody did not show the expected labelling pattern with any of the described procedures, it was considered as unsuitable for IHC on paraffin wax sections.

Optimized IHC conditions were as follows. Antigen retrieval was carried out by boiling the slides in Tris/EDTA buffer in a conventional microwave oven at 750W for 20 min. The sections were allowed to cool for 10 min and were then incubated with the primary antibody for 1 h at room temperature. For both cell pellets and tissues, the following dilutions were used: CM-1 Signet 1 in 40, CM-1 Novocastra 1 in 150, PAb240 1 in 25, PAb122 1 in 10. Endogenous peroxidase was inactivated by immersing the slides in peroxidase blocking solution (Dako Cytomation) for 10 min at room temperature. For detection of the primary antibody, the Detection or EnVision Kit (Dako Cytomation) was applied according to the manufacturer's instructions. Finally, the reaction was “visualized” by an AEC chromogen (Dako Cytomation). All immunolabelling of tissues was performed on arrays and, if required for verification, additionally on sections from the donor blocks. The slides were counterstained with haemalum. Samples were considered positive when either at least 10% of the neoplastic cells in a tumour or a subpopulation of cells within a normal tissue showed distinct intranuclear labelling.

Immunohistochemistry on formalin-fixed paraffin wax-embedded bacterial pellets was performed as described above. Additionally, the primary antibody was progressively diluted until the difference between bacteria expressing both GST and p53 and those expressing GST only disappeared. The dilution steps were as follows 1 in 10, 25, 50, 100, 250, 500, 1000, 2500, 5000, 10 000, 20 000 and 40 000. The highest dilution showing a clear-cut difference between p53-positive and -negative bacteria was defined as the antibody titre.

Mutational Analysis of the TP53 Gene in Selected Samples

To facilitate interpretation of the immunohistochemical findings, a confirmatory mutational analysis of exons 5 to 10 of the TP53 gene was performed with DNA extracts from canine tumour samples showing positive immunohistochemical results for p53. These samples included lymphomas 1 to 5 and the canine mammary carcinoma indicated in Table 2. Osteosarcoma samples 1 and 2 were excluded because of their small size.

DNA extraction from paraffin wax blocks. DNA from the tumour samples was retrieved after trimming the original block from normal tissue to yield only tumour tissue. Three 30-μm sections were cut and collected in an Eppendorff tube. DNA extraction was performed with the DNeasy Blood and Tissue Extraction Kit (Quiagen GmBH, Hilden, Germany) according to the manufacturer's instructions. Lysis was carried out overnight at 55 °C in a shaker. The end product was eluted in 200 μl of AE buffer (provided with the Kit).

PCR amplification of p53 exons 5 to 10. A 5-μl volume of elution product was used to amplify each exon in a separate reaction, with Taq Gold Polymerase (Invitrogen) and the following stated primers. Exon 5: (fwd) 5'-cgacccctctacttctcacc-3', (rev.) 5'-agccttgtcccatctgtagc-3'. Exon 6: (fwd) 5'-agatgggacaaggctgctc-3', (rev.) 5'-ctcccagagacccctcagat-3'. Exon 7: (fwd) 5'-tgggaaagactgaggctgat-3', (rev.) 5'-cagtaaggaagtggccagga-3'. Exon 8: (fwd) 5'-atgagggtggctaggagtca-3', (rev.) 5'-cctcctcttgtcttgcttgc-3'. Exon 9: (fwd) 5'-acagagggtgcaattctgct-3', (rev.) 5'-ctagcctcacatgtgctcca-3'. Exon 10: (fwd) 5'-gtggcttccccctcttattc-3', (rev.) 5'-gaatcctgtggctttccaac-3'. Cycling conditions were 95 °C for 10 min followed by 38 cycles of denaturing for 50 sec at 94 °C, annealing for 50 sec at a temperature adjusted for each primer pair, and polymerization for 1 min at 72 °C. The final extension step was 10 min at 72 °C. The PCR products yielded sizes between 207

and 293 base pairs. They were purified with the High Pure PCR Product Purification Kit (Roche), according to the manufacturer's instructions, and sent to a commercial company (Microsynth AG, Balgach, Switzerland) for sequencing (cycle sequencing) with the forward primer. The chromatograms provided by the company were analysed manually, the sequences obtained were searched for mutations by aligning with the dog genome sequence. For this purpose the BLAST tool available at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) was used. In the case of single unclear sequences or of a mutation, PCR and sequencing were repeated.

Results

Testing Cross-reactivity of Antibodies with Recombinant Canine p53

The following five commercially available antibodies, raised against human p53 for use in IHC, were selected: DO-12, DO-13, PAb122, PAb240 (all monoclonal) and CM-1 (polyclonal). Criteria for selection were a high degree of sequence homology of the immunogen peptide with the corresponding canine epitopes, or previously reported use (Table 1) or both. Cross-reactivity of the antibodies with recombinant canine p53 was then determined. All antibodies specifically recognized a GST-p53 fusion protein in western blots of bacterial lysates by labelling a band of the expected molecular weight (Fig. 1a). No labelling was seen in the blots of lysates from GST-protein-expressing control bacteria. As an exception, antibody CM-1 showed two bands with a molecular weight lower than that of the fusion protein, probably representing cross-reaction with bacterial antigens. All lysates were initially tested for GST expression by detecting a band of the expected molecular weight by immunoblotting with a GST-specific antibody (data not shown). In addition, all antibodies immunolabelled formalin-fixed, paraffin wax-embedded pellets consisting of bacteria expressing fusion protein (Fig. 1b). The difference in signal intensity between p53-positive and -negative pellets elicited by the monoclonal antibodies was marked. The titres (defined as the highest antibody dilution retaining a clear-cut difference in labelling between positive and negative pellets) were 1 in 20 000 for PAb240, 1 in 2500 for PAb122, and 1 in 250 for DO-12 and DO-13. In contrast, with

polyclonal antibody CM-1 the difference in labelling between positive and negative pellets was weak and difficult to reproduce, due to a variable degree of concomitant staining of p53-negative pellets. An additional monoclonal antibody (clone DO-7) failed to immunolabel either positive or negative bacterial pellets (data not shown) and was therefore not further used in this study.

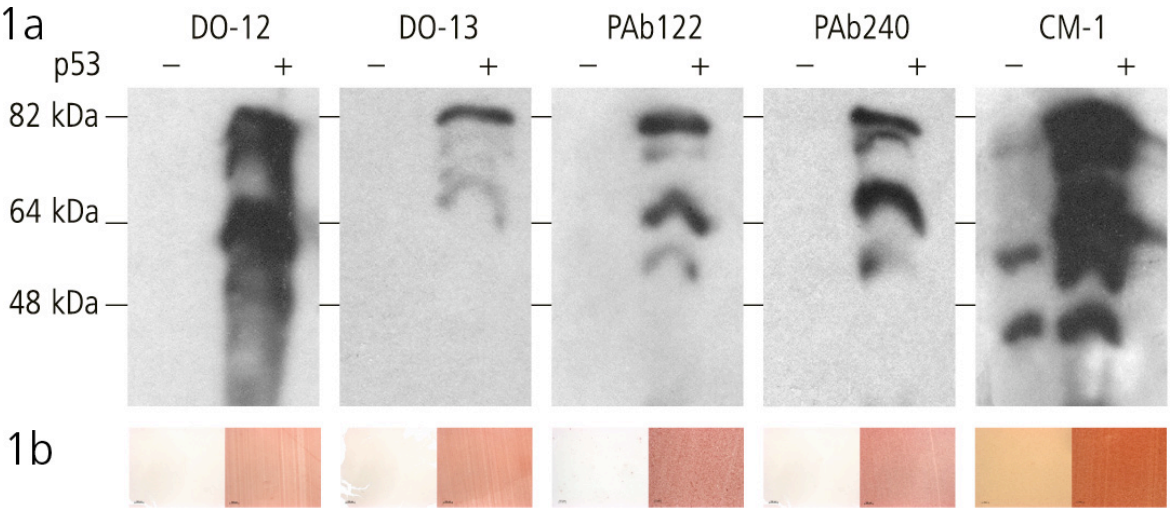

Fig. 1a, b. Testing of human-to-canine cross-reactivity of five p53 antibodies. (a) Western blot analysis of *E. coli* lysates expressing either GST-tagged canine p53 (+) or GST alone (-). All antibodies label a band of the expected molecular weight for the fusion protein. (b) Immunohistochemistry of formalin-fixed and paraffin wax-embedded bacterial pellets expressing either GST-tagged canine p53 (+) or GST alone (-). Monoclonal antibodies clearly label p53-expressing bacteria; polyclonal antibody CM-1 less clearly discriminates between p53-expressing and tag-only-expressing bacteria.

In-vitro Apoptosis Model with Cultured Canine Keratinocytes

An *in-vitro* model was developed to determine whether the selected antibodies were suitable for the immunohistochemical detection of endogenous p53 in normal canine tissue. Canine keratinocytes were irradiated with UVB to induce apoptosis, which under such circumstances is mediated mainly by stabilization of p53 (Smith and Fornace, 1997). The resulting increase of total p53 protein renders it detectable by IHC, while protein concentrations are below detection level in the majority of untreated cells.

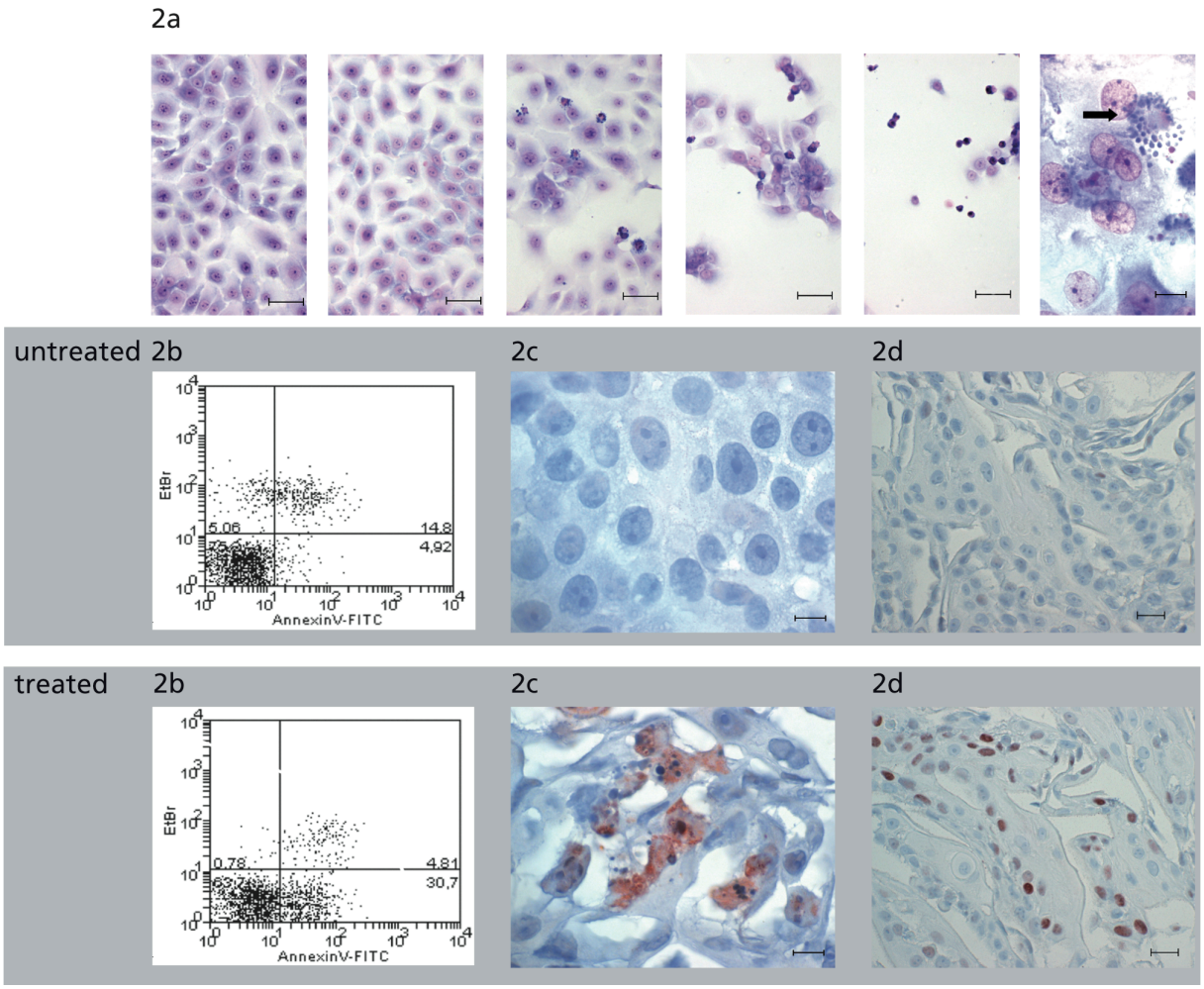

Fig. 2a-d. UVB-induced apoptosis in canine keratinocytes. (a) Giemsa staining (from left to right: untreated control and 6, 12, 18 and 24 h after irradiation), showing increasing monolayer disruption and apoptosis. Bars, 50 μm; the 6th image (far right) shows detail of apoptotic cells (arrow). Bar, 10 μm. (b) Flow cytometry analysis (untreated and treated cells 24 h after irradiation), showing significant increase in Annexin V-positive and ethidium bromide (EtBr)-negative portion in treated cells. (c) Immunohistochemistry for active caspase-3 (untreated and treated cells 24 h after irradiation); treated, apoptotic cells are strongly labelled, whereas healthy cells are negative. Bars, 10 μm. (d) Immunohistochemistry for p53 (clone PAb240, untreated and treated cells 6 h after irradiation); the signal is restricted to the nucleus of treated cells and is homogeneously spread over the whole nuclear section area. Labelled cells are distributed in patches. Bars, 20 μm.

Successful induction of apoptosis was demonstrated morphologically in Giemsa-stained monolayers, by Annexin V detection in flow cytometric analysis and

by the immunohistochemical detection of cleaved caspase-3. As determined by light microscopy of Giemsa-stained monolayers (Fig. 2a), 6 h after irradiation no signs of apoptosis were observable. However, 12 h after UV irradiation the monolayer was interspersed with apoptotic bodies as well as cells showing fragmented nuclei and intense membrane blebbing. As a result, occasional gaps appeared between the cells. Six hours later the monolayer was disrupted and large numbers of apoptotic cells were visible. Twenty-four hours after irradiation, numerous apoptotic cells were still present, interspersed with small patches of unaffected cells. Annexin V detection in flow cytometric analysis confirmed the apoptotic nature of the cell death observed. Twenty-four hours after treatment about 30% of the gated cells showed positive staining for Annexin V while being negative for ethidium bromide. In contrast, only 5% of gated untreated cells were apoptotic (Fig. 2b). Immunolabelling of formalin-fixed and paraffin wax-embedded irradiated cell pellets with an antibody specific for active caspase-3 clearly demonstrated cells with apoptotic morphology and occasional cells with unaltered appearance (Fig. 2c). In cells showing early apoptotic changes, the signal was restricted to the cytoplasm. However, with progressing apoptosis and disintegration of the nucleus, the cells appeared as homogeneously labelled, round bodies with occasional dark chromatin clumps. A small number of UV-irradiated cells with apoptotic morphology, as well as the untreated cells, were completely devoid of active caspase-3 staining.

Of the five anti-p53 antibodies tested, PAb122, PAb240 and CM-1 (one batch each from two different vendors) showed a labelling pattern compatible with p53 stabilization in irradiated, paraffin wax-embedded cells starting 6 h after treatment and still visible in non-apoptotic cells 24 h after treatment (Fig. 2d and Table 2). The signal was restricted to the nucleus and was homogeneously spread over the whole nuclear area. Positive cells were distributed in patches, probably reflecting varying degrees of p53 stabilization due to differences in cell cycle stage in individual cells. The untreated control showed a few isolated, mainly weakly labelled, cells scattered throughout the section. In contrast, no specific signal was detected with antibodies DO-12 and DO-13. It was noteworthy that two additional batches of polyclonal antibody CM-1 from the same vendors elicited discrepant results. One antibody produced no labelling, while the second showed a slight increase in the number of positive nuclei with a labelling pattern differing from that of all other antibodies in two

consecutive experiments. In these cells, the signal had a granular appearance and spared some areas of the nucleus, especially the nucleoli.

Table 2: Overview of p53 antibody reactivity

	In-vitro	Tumour tissues with positive reaction*									Normal
	irradiated	Human	Canine								canine
	canine		lymphomas					osteosarcomas		mammary	tissues
Antibody	cells		1	2	3	4	5	1	2	carcinoma	
DO-12	-	+	-	-	-	-	-	-	-	-	-
DO-13	-	+	-	n.d.	n.d.	n.d.	n.d.	-	-	-	n.d.
PAb122	+	+	-	-	-	-	+	+	+	-	-
PAb240	+	+	-	-	-	-	+	+	+	-	-
CM-1†	-	+	+	+	+	+	-	-	-	+	+
CM-1‡	+	+	-	-	-	-	+	+	+	-	-
CM-1§	+/-	+	+	+	+	+	-	-	-	+	+
CM-1¶	+	+	-	-	-	-	+	+	+	-	-

* Only positive cases out of 24 lymphomas, 4 osteosarcomas, 4 mammary carcinomas, 5 fibrosarcomas and 5 seminomas are included. Positive lymphomas and positive osteosarcomas were numbered separately

+, Positive reaction (irradiated cells: significant increase in number of positive cells compared to negative control cells; tumours: >10% positive cells; normal canine tissues: isolated cells labelled).

+/-, Slightly increased number of positive cells but atypical intranuclear labelling pattern. -, Negative reaction.

† Novocastra, Lot No. 300507; ‡ Novocastra, Lot No. 300509; § Signet, Lot No. 05DC00654; ¶ Signet, Lot No. 05LC02510.

n.d., Not done.

IHC of Formalin-fixed, Paraffin Wax-embedded Tissues

The next step was to explore whether cross-reacting antibodies as determined above were suitable for use on formalin-fixed, paraffin wax-embedded, canine normal and neoplastic tissues. The samples, which were included in tissue arrays, consisted of human tissues with known p53 status, a panel of non-neoplastic tissues from three different dogs, and a panel of different canine tumours. All antibodies labelled the human controls as expected but did not label canine normal tissues with the few exceptions indicated below (Fig. 3b). Clones DO-12 and DO-13 produced only non-specific cytoplasmic labelling in the absence of a nuclear signal in canine tumours. Antibodies PAb122, PAb240, and CM-1 (two batches purchased from two different vendors) all labelled two out of three osteosarcomas (Fig. 3c)(all cores of the fourth

osteosarcoma yielding only necrotic tissue and therefore being excluded from the evaluation), one of 24 malignant lymphomas (Fig.3a), and none of four mammary adenocarcinomas, five fibrosarcomas and five seminomas (Table 2). In all positive tumours over 50% of the cells were positive. In general, PAb122 generated a weaker signal and smaller numbers of positive cells than did the other antibodies (Fig. 3c). No significant unspecific intranuclear labelling was detected in the normal canine control tissues with any of the three monoclonal antibodies. Occasionally, however, weak intracytoplasmic background staining was present with the polyclonal CM-1 antibodies.

The two additional batches of polyclonal antibody CM-1 yielded different results, which are described and commented on in detail below. Both batches elicited an intranuclear signal in a different subset of the canine tumours, including four of 24 malignant lymphomas (Fig. 3a) and one of four mammary adenocarcinomas (Table 2). All other neoplastic tissues were clearly negative; lymphoma case 5 showed faint intranuclear labelling, which was considered negative. Notably, the antibodies labelled the human positive control tissues, but the results with cultured canine positive control cells were not satisfactory, as indicated above. In addition, these antibodies also produced intranuclear labelling of cells in a lymph node with follicular hyperplasia included in the normal canine tissue panel (Fig. 3b). Labelled cells were located within both the germinal centre and the sinus regions. They included spindle cells with elongated nuclei and macrophages, thus appearing to belong to different lineages. All other normal canine tissues exhibited no significant labelling with these antibodies. In lymphomas 1 and 2, and in one mammary adenocarcinoma, labelled cells were considered neoplastic on the basis of their morphology. In lymphomas 3 and 4, additional cell types were labelled, including cells with elongated nuclei and macrophages. To exclude an artefact associated with the tissue array technique, sections from the original tissue blocks of lymphomas 1 to 5 and the positive mammary adenocarcinoma were labelled. The results accorded with the findings described above. It became evident that positive cells in lymphomas 3 and 4 were mainly restricted to peripheral areas of the tumour tissues and to a considerable extent consisted of non-neoplastic cells. Positive tumour cells were distributed in clusters in lymphoma 2 and evenly in lymphoma 1 and in the positive mammary adenocarcinoma. Finally, the results with all CM-1 antibody batches shown in Table 2

were basically confirmed when citrate buffer was used instead of Tris/EDTA for antigen retrieval, although the signal was less intense (data not shown).

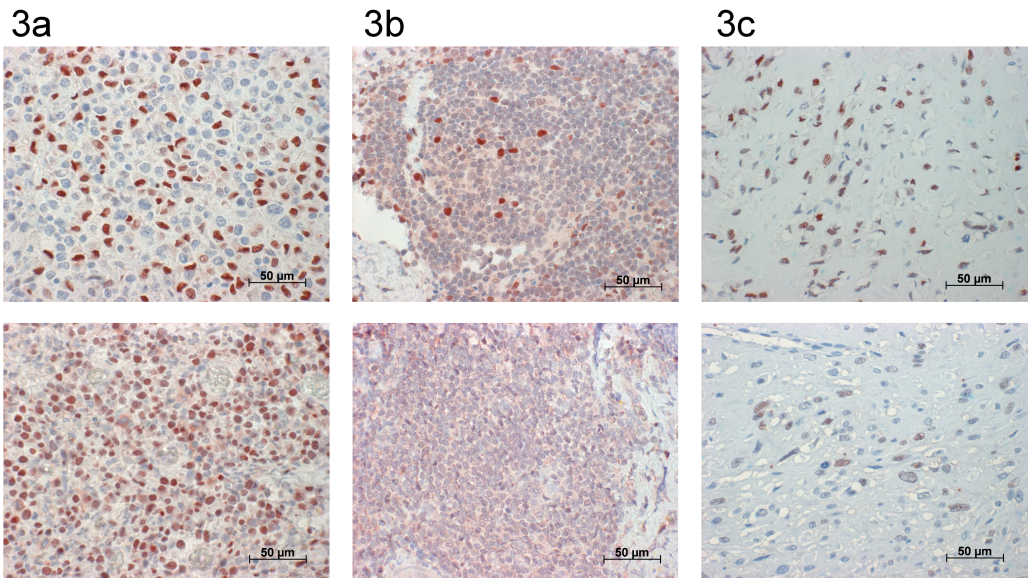

Fig. 3a-c. Immunohistochemistry (p53) of canine tissues. (a) Lymphoma no. 1 (CM-1 Signet Lot No. 05DC00654 [top]) and lymphoma no. 5 (CM-1 Signet Lot No. 05LC02510 [bottom]); intense intranuclear labelling of cells with morphology of neoplastic lymphocytes. (b) Lymph node with follicular hyperplasia (CM-1 Signet Lot No. 05DC00654 [top], and CM-1 Signet Lot No. 05LC02510 [bottom]); non-specific intranuclear reaction with one batch of polyclonal antibody CM-1. (c) Osteosarcoma no. 1 (PAb240 [top] and PAb122 [bottom]); stronger labelling with PAb240 than with PAb122.

Mutational Analysis of the TP53 Gene in Selected Samples

DNA of sufficiently good quality for PCR amplification was retrieved from tumour tissues in lymphoma cases 1 to 5 but not from the mammary carcinoma. All sequences of all exons examined were wild type except for exon 8 of lymphoma 5, which displayed a mutation (A instead of G) at base 868 of the coding sequence (starting from the ATG), resulting in a single amino-acid substitution (R instead of G) at codon 290.

Discussion

A system was devised for selecting p53-specific antibodies for IHC on canine tissues. First, human-to-canine antibody cross-reactivity was tested in respect of recombinant canine p53 protein. Western blot analysis and IHC on embedded bacteria yielded similar results. However, the latter method was simpler, faster, and more closely related to the final application, and worked well with monoclonal antibodies. This procedure revealed that clone DO-7 did not cross-react with canine p53. In a previous study this antibody failed to label putative p53-positive canine tissues (Albaric *et al*., 2001), and data derived from the present study suggest that the targeted epitope is missing in the canine molecule. All remaining monoclonal antibodies in the present study showed specific labelling of p53-expressing bacteria up to the dilution defined as the titre. Moreover, the titre may predict to some extent whether an antibody is suitable for tissue IHC, since antibodies with a low titre worked less well, if at all, than those with a higher titre. In contrast, polyclonal antibody CM-1 was less suitable for use with embedded bacteria. This finding may be attributable to the fact that this antibody had not been preabsorbed, and probably contained moieties directed against bacterial antigens, as was suggested by unspecific bands in western blots of GST-only-expressing bacterial lysates. Nevertheless, antibody CM-1 specifically labelled recombinant p53 in western blots, indicating that it indeed recognized canine p53. Western blotting may therefore be better than IHC of embedded bacteria for testing polyclonal antibodies for interspecies cross-reactivity. In conclusion, this part of the study indicated that most commercially available antibodies against p53 can be expected to cross-react to some extent with canine p53. Additional fusion proteins of p53 isoforms or other family members would improve this system. In general, a selection procedure based on recombinant protein, as described, has the advantage of unequivocally determining cross-reactivity; this may be of particular importance when specific tissue controls are not available.

The second step addressed the question of whether the selected antibodies were suitable for immunohistochemical detection of endogenous p53 in normal canine tissues. UVB-irradiated normal canine keratinocytes were used in view of abundant published data indicating that cell death is mainly p53-dependent in this setting (Smith and Fornace, 1997). Apoptosis was demonstrated by three different criteria. Only three of the five antibodies cross-reacting with canine p53 (PAb122, PAb240 and CM-1) enabled a p53 increase to be detected by IHC in UV-irradiated

cells. These three antibodies gave consistent results in tumour tissues, indicating the suitability of the cell culture control system. Furthermore, the cell culture experiments made it possible to discriminate between different batches of polyclonal antibody CM-1, supporting the notion that the labelling differences observed with additional batches of CM-1 may have been artefactual. This was further substantiated by the results of the mutational analysis. Antibody PAb240 is often claimed to be specific for mutated p53, probably due to a conformational change of the protein leading to the exposure of the relevant epitope (Gannon *et al*., 1990). The data from the present cell culture experiments indicate that PAb240 in addition labels canine wild-type p53. This may be associated with the antigen retrieval procedure, which may cause the protein to denature, thus exposing the specific epitope; alternatively, it may reflect conformational differences between human and canine p53.

Thus, the cell culture system used appeared to be a reliable screening method for antibodies against p53. Furthermore, it might prove useful for testing antibodies against other constituents of the apoptotic pathways. In support of this, the present data indicate that the antibody against active caspase-3 used to characterize the cell culture model may be suitable for IHC of canine tissues, as suggested by a previous study with uterine samples (Van Cruchten *et al*., 2003).

The next step in this study was to investigate whether the selected antibodies were suitable for the immunohistochemical detection of p53 in tissues. In general, the results from tumour tissues accorded with those from cell cultures, and the sensitivity of the antibodies accorded with the titres determined with bacterial pellets. PAb122 appeared to be less sensitive than PAb240 and CM-1, and clones DO-12 and DO-13 appeared to be unsuitable for the immunohistochemical detection of canine p53. Overall, a surprisingly small number of positive tissues was found in the collection of canine tumours examined; this may have been due in part to the relatively small number of samples, to the limited area of tumour tissue examined in the tissue arrays, or to insufficient sensitivity of the antibodies or detection system. The results with osteosarcomas were consistent with previous findings, in which frequent p53 alterations were reported in this tumour type (Johnson *et al*., 1998). In fibrosarcomas, an intermediate proportion of positive cases may be expected, as suggested by a study in which six positives were identified in 17 (35%) samples (Gamblin *et al*., 1997). The proportion of positive mammary adenocarcinomas greatly varies between studies (Gamblin *et al*., 1997; Schafer *et al*., 1998; Rungsipipat *et al*., 1999; Inoue

and Shiramizu, 1999; Haga *et al*., 2001; Wakui *et al*., 2001; Pena *et al*., 2003; Lee *et al*., 2004). All five seminoma samples in the collection were negative, in contrast to two previous investigations reporting immunohistochemical detection in 100% of canine seminomas (Vitellozzi *et al*., 1998; Inoue and Wada, 2000). Different antibodies were used in each of these two earlier studies, and in one of them a large portion of normal spermatocytes were also labelled. However, data from human studies suggest that such tumours frequently express wild-type p53 at concentrations detectable by IHC (Houldsworth *et al.*, 1998). This discrepancy warrants further study.

Finally, the results point to a low proportion of p53-positive results in canine lymphomas. Thus, Gamblin *et al*. (1997) and Sokolowska *et al*. (2005) reported 40% of positives (six out of 15, and 21 out of 52 tumours, respectively), while Sueiro *et al*. (2004) found no positives out of 28. In this type of neoplasm, factors such as non-homogeneous distribution of positive cells and the relative numbers of neoplastic versus non-neoplastic cells may play a role. In addition, the cut-off chosen to distinguish possible mutation-positive from mutation-negative tumours (i.e., the percentage of positive cells) may be important. Most authors in human and veterinary studies set this parameter at 10%. However, others suggest that a cut-off of at least 20% is more appropriate (Ishida *et al*., 1997; Gao *et al*., 2000; Cruz *et al*., 2002; Zettl *et al*., 2003). A further important determinant for the cut-off is the sensitivity of the antibody used (Pardo *et al*., 2004). Antibodies such as PAb122, PAb240 or CM-1 have been shown in human studies to be less sensitive and of lesser prognostic relevance than newer monoclonal antibodies (Baas *et al*., 1994; Xu *et al*., 1994). Currently, most human medical studies use monoclonal antibodies directed against the amino terminus of p53, which has been shown to contain a large portion of the immunodominant epitopes (Schlichtholz *et al*., 1994; Munro *et al*., 2005). Even CM-1 appears to be mainly directed against such epitopes in human p53, since it was found to recognize full-length p53 but not Δ133p53, a variant lacking the first 133 N-terminal amino acids (Bourdon *et al*., 2005). Unfortunately, the N-terminus of canine p53 is the region displaying the least homology with the human counterpart. In the light of the present results and of the considerations above it appears that canine specific antibodies would significantly improve the quality of p53 IHC in canine tissues. However, this would not make mutational analysis dispensable if all mutations were to be detected.

In conclusion, a test system is described for the selection of specific antibodies for use in IHC. The system consists of three steps, each of which has intrinsic advantages, as shown by the example of canine p53. The use of recombinant canine protein facilitates the exclusion of non-cross-reacting antibodies. Cell cultures with controlled antigen expression are valuable in checking the antibodies immunohistochemically in a homologous system and enabling those with low sensitivity to be excluded. Homologous tissues with known high and low expression status are of great help in substantiating further the suitability of the antibodies. Positive human tissues are unsuitable as controls for IHC of canine tissues, but appropriate positive and negative controls are indispensable, even in cases of a protein with an "obvious" labelling pattern such as p53. Particular attention should be paid to this point, especially when polyclonal antibodies raised against heterologous antigens are used, since significant differences may occur between batches.

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Curriculum Vitae

Name:	Stefan Matthias Keller
Geburtsdatum:	12.12.1975
Geburtsort:	Karlsruhe
Nationalität:	Deutsch

1982-1986	Grundschule Waldbronn, Deutschland
1986-1992	Gymnasium Karsbad, Deutschland
1992-1993	South Kitsap High School, Port Orchard, Washington, USA
1993-1995	Gymnasium Karsbad, Deutschland; Abschluß: Abitur
1996-1997	Zivildienst, Schule für Körperbehinderte, Karlsbad, Deutschland

1997-2003	Studium der Veterinärmedizin an der Freien Universität, Berlin, Deutschland
2003	3. Staatsexamen an der Freien Universität Berlin, Deutschland

2004-2007	Doktorand am Institut für Veterinärpathologie, Vetsuisse-Fakultät, Universität Zürich, Schweiz

Zürich, 10.6.2007